Proteolytic Processing Yields Two Secreted Forms of Sonic hedgehog

DAVID A. BUMCROT, RITSUKO TAKADA, AND ANDREW P. McMAHON*

Department of Molecular and Cellular Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

sonic hedgehog (*Shh*) is expressed in tissues with known signalling capacities, such as the notochord, the floor plate of the central nervous system, and the zone of polarizing activity in the limb. Several lines of evidence indicate that Shh is involved in floor plate induction, somite patterning, and regulation of anterior-posterior polarity in the vertebrate limb. In this report, we investigate the biochemical behavior of Shh in a variety of expression systems and embryonic tissues. Expression of mouse Shh in *Xenopus* oocytes, COS cells, and baculovirus-infected insect cells demonstrates that in addition to signal peptide cleavage and N-linked glycosylation, chicken and mouse Shh proteins undergo additional proteolytic processing to yield two peptides with molecular masses of approximately 19 kDa (amino terminus) and 27 kDa (carboxy terminus), both of which are secreted. In transfected COS cells, we show that the 19-kDa peptide does not accumulate significantly in the medium unless heparin or suramin is added, suggesting that this peptide associates with the cell surface or extracellular matrix. This retention appears to depend on sequences in the carboxy-terminal part of the peptide. Finally, detection of the 19-kDa product in a variety of mouse and chicken embryonic tissues demonstrates that the proteolytic processing observed in cell culture is a normal aspect of Shh processing in embryonic development. These results raise the possibility that amino- and carboxyl-terminal regions of Shh may have distinct functions in regulating cell-cell interactions in the vertebrate embryo.

The determination of cell fate during embryonic development depends in large part on communication between distinct cell populations. Often, this communication is mediated by diffusible protein factors which emanate from signalling centers and are interpreted by responsive cells. Although this concept was initially described nearly a century ago, it is only recently that candidate molecules capable of performing these functions have begun to be identified.

One well-studied instance of signalling in development is the establishment of doral-ventral patterning in the vertebrate central nervous system (CNS) (for reviews, see references 15, 37, and 39). At the neural plate stage, signals from the notochord instruct midline cells of the overlying neural ectoderm to form a structure known as the floor plate (32, 41, 52). This induction appears to require contact between the inducing and responding tissues (31). Subsequently, the notochord and floor plate induce the formation of motor neurons in a ventral lateral region of the neural tube (33, 51). In contrast to floor plate induction, the induction of motor neurons is not contact dependent, since medium conditioned by cultured notochord and floor plate explants is able to induce motor neurons (51). Furthermore, midline signals from the floor plate and notochord are necessary for the induction of sclerotomal development in the adjacent ventral somite (4, 16, 36). Thus, local acting and diffusible signals are thought to mediate the development of ventral polarity within the CNS and somite.

The establishment of the anterior-posterior axis in the developing limb provides another well-studied example of signalling in the regulation of axial polarity (for reviews, see references 19 and 45). Correct digit identity has been shown to depend on a region of posterior limb bud mesenchyme known as the zone of polarizing activity (ZPA). Grafting portions of the ZPA to the anterior side of host limb buds leads to reprogramming of the anterior mesenchyme, resulting in the formation of a second limb with mirror image symmetry relative to the normal anterior-posterior axis (38, 49, 53). This result indicates that the ZPA is essential for specifying posterior identity and, together with other grafting experiments, has led to a model in which the ZPA is thought to be the source of a morphogen gradient controlling anterior-posterior polarity in the limb (50).

Recently, members of the *Hedgehog* gene family have emerged as candidates for both notochord-floor plate- and ZPA-signalling activities (for reviews, see references 9, 13, and 40). *Hedgehog* was initially identified as a member of the segment polarity class of *Drosophila* pattern-regulating genes (29). Subsequent analysis has demonstrated that *Drosophila Hedgehog* endcodes a secreted protein which plays a major role in patterning the insect segment, as well as eye, leg, and wing imaginal discs (for a review, see reference 30). It is also likely to regulate many other aspects of larval and pupal development (1, 22, 27, 43, 47, 48).

Vertebrate *Hedgehog* homologs have been identified in mice (5, 6), rats (35), chickens (34), zebra fish (18), and humans (5, 23), with multiple family members identified in some species. One family member, *sonic hedgehog* (*Shh* [also known as *vhh-1* or *Hhg-1*]), is expressed in the notochord, floor plate, and posterior limb bud mesenchyme (5, 6, 18, 34, 35). Furthermore, it has been demonstrated that misexpression of *Shh* in a dorsal region of the mouse neural tube leads to ectopic activation of ventral tissue-specific genes (6), as does ubiquitous expression of *Shh* in frog or zebra fish embryos (18, 35). In addition, ectopic expression of *Shh* in the anterior limb mesenchyme leads to digit duplications reminiscent of ZPA grafts (5, 20, 34) and can activate sclerotomal gene expression in the paraxial mesoderm (8, 16).

These experiments have established that Shh is likely to be a

* Corresponding author. Mailing address: Department of Molecular and Cellular Biology, The Biological Laboratories, Harvard University, 16 Divinity Ave., Cambridge, MA 02138. Phone: (617) 496-3757. Fax: (617) 496-3763.

mediator of signalling activities that pattern the CNS, limb, and somites; however, they pose a number of questions as to how this signal operates. In order to explore the mode of action of *Shh*, we have investigated the biochemical behavior of the Shh protein. In this report, we demonstrate that mouse Shh is a glycoprotein that is proteolytically cleaved into two smaller forms, both of which are secreted. Similar processing of Shh is shown to occur in a variety of expression systems and in embryonic tissues. Cleavage is predicted to generate a highly conserved amino-terminal peptide consisting primarily of sequences encoded by exons 1 and 2 and a less well-conserved carboxy-terminal peptide encoded mostly by exon 3. Accumulation of the amino-terminal peptide in the medium of expressing cells is greatly increased by treatment with heparin or suramin, suggesting that this peptide associates with the cell surface or extracellular matrix (ECM). These data are consistent with a role for hh in cell-cell communication during vertebrate development and raise the possibility that distinct activities may reside in different regions of the Shh protein.

MATERIALS AND METHODS

In vitro translation and processing. Mouse and chicken *Shh* coding sequences were inserted into the vector pSP64T (kindly provided by D. Melton), which contains an SP6 phage promoter and both 5′ and 3′ untranslated sequences derived from the *Xenopus laevis* β-globin gene. After restriction endonuclease digestion with *Sal*I to generate linear templates, RNA was transcribed in vitro using SP6 RNA polymerase (Promega, Inc.) in the presence of 1 mM cap structure analog [m^{7}G(5′)ppp(5′)Gm; Boehringer Mannheim, Inc.]. Following digestion with RQ1 DNase I (Promega, Inc.) to remove the DNA template, transcripts were purified by phenol-chloroform extraction and ethanol precipitation.

Rabbit reticulocyte lysate (Promega, Inc.) was used according to the manufacturer's instructions. For each reaction, 12.5 μl of lysate was programmed with 0.5 to 2.0 μg of in vitro-transcribed RNA. The reaction mixtures contained 20 μCi of Express labeling mix (NEN/DuPont, Inc.). To address processing and secretion in vitro, 1.0 to 2.0 μl of canine pancreatic microsomal membranes (Promega, Inc.) was included in the reaction mixtures. The final reaction volume of 25 μl was incubated for 1 h at 30°C. Aliquots of each reaction mixture (between 0.25 and 3.0 μl) were boiled for 3 min in Laemmli sample buffer (LSB; 125 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 0.25 mg of bromophenol blue per ml) before separation on a 15% polyacrylamide gel. Fixed gels were processed for fluorography using En^{3}Hance (NEN/DuPont, Inc.) as described by the manufacturer.

Glycosylation was addressed by incubation with endoglycosidase H (endo H; New England Biolabs, Inc.) according to the manufacturer's directions. Reactions were carried out for 1 to 2 h at 37°C before reaction products were analyzed by polyacrylamide gel electrophoresis (PAGE).

***Xenopus* oocyte injection and labeling.** Oocytes were enzymatically defolliculated and rinsed with OR2 (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.2], 82 mM NaCl, 2.5 mM KCl, 1.5 mM Na$_2$HPO$_4$). Healthy stage six oocytes were injected with 30 ng of in vitro-transcribed, capped mouse *Shh* RNA (prepared as described above). Following a 2-h recovery period, healthy injected oocytes and uninjected controls were cultured at room temperature in groups of 10 in 96-well dishes containing 0.2 ml of OR2 (supplemented with 0.1 mg of gentamicin and 0.4 mg of bovine serum albumin [BSA] per ml) per well. The incubation medium was supplemented with 50 μCi of Express labeling mix. Three days after injection, the culture media were collected and expression of Shh protein was analyzed by immunoprecipitation. Oocytes were rinsed several times in OR2 before being lysed in TENT (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with 1 μg of aprotinin and 2 μg of leupeptin per ml and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 13,000 × g for 10 min at 4°C, soluble protein supernatants were recovered and analyzed by immunoprecipitation (see below).

COS cell transfection and labeling. COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, Inc.) supplemented with 10% fetal bovine serum (Gibco/BRL), 2 mM L-glutamine (Gibco/BRL), and 50 mU of penicillin and 50 μg of streptomycin (Gibco/BRL) per ml. Subconfluent COS cells in 35- or 60-mm dishes (Falcon, Inc.) were transiently transfected with 2 or 6 μg of supercoiled plasmid DNA, respectively. Between 42 and 44 h posttransfection, the cells were labelled for 4 to 6 h in 0.5 (35-mm dishes) or 1.5 (60-mm dishes) ml of serum-free DMEM lacking cysteine and methionine (Gibco/BRL) and supplemented with 125 μCi each of Express labeling mix and [L-^{35}S]cysteine (NEN/DuPont) per ml. After labeling, media were collected and used for immunoprecipitation. Cells were rinsed with cold phosphate-buffered saline (PBS) and lysed in the tissue culture dishes by the addition of 0.5 (35-mm dishes) or 1.5 (60-mm dishes) ml of TENT (with protease inhibitors as described above) and

gentle rocking for 30 min at 4°C. The lysates were cleared by centrifugation (13,000 × g for 5 min at 4°C), and the supernatants were analyzed by immunoprecipitation (see below).

For the experiments involving polyanionic medium additives, heparin (Sigma) and suramin (Miles, Inc.) stocks were prepared by dissolving in DMEM at concentrations of 100 mg/ml and 200 mM, respectively. Between 42 and 44 h after transfection, cells in 35-mm dishes were rinsed with PBS and refed with 0.5 ml of serum-free DMEM containing different concentrations of these additives. Following incubation for 4 h, culture media were collected and cleared of cells by centrifugation. The cells were rinsed with cold PBS and lysed in the dishes by the addition of 0.1 ml of LSB. The samples were analyzed by Western blotting (immunoblotting) (see below).

Baculovirus production and infection. A recombinant baculovirus expressing mouse *Sonic hedgehog* with a Myc epitope tag inserted at the carboxy terminus was generated using the Baculogold kit (Pharmingen, Inc.). The initial virus production used Sf9 cells, after which two rounds of amplification in High Five cells (Invitrogen, Inc.) in serum-free medium (ExCell 401; JRH, Inc.) were performed. A baculovirus lacking *Shh* coding sequences was also constructed as a control. For protein induction, High Five cells were infected at a multiplicity of approximately 15. Three days later, medium and cells were collected by gentle pipetting. Cells were collected by centrifugation (1,000 × g), and the medium was recovered for Western blot analysis. Cell pellets were washed twice in cold PBS and lysed in TENT plus protease inhibitors (see above) by rotation for 30 min at 4°C in a microcentrifuge tube. The lysate was cleared as described above prior to Western blotting.

Western blotting. For Western blotting of lysates and media from baculovirally infected cells, 0.25-ml samples of media (1% of the total) were precipitated with trichloroacetic acid (TCA) and redissolved in 15 μl of LSB. Cell lysate samples (1% of total) were brought to a final volume of 15 μl with water and 5× concentrated LSB. Samples were boiled for 5 min prior to separation on a 15% acrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Inc.) and blocked in BLOTTO–0.2% Tween 20 (11). Hybridoma supernatant recognizing the human c-Myc epitope (9E10 [7]) was added at a dilution of 1:200 for 1 h, which was followed by a 1:5,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Promega, Inc.) for 30 min. Bands were visualized by using the Lumi-Phos 530 reagent (Boehringer Mannheim) according to the manufacturer's directions.

For Western blotting of COS cell material, cleared media (see above) were precipitated with TCA in the presence of 4 μg of BSA per ml as a carrier. The protein pellets were dissolved in 20 μl of LSB. Dissolved medium protein and cell lysates (see above) were boiled for 5 min, and 10 μl (50%) of each medium sample and 10 μl (10%) of each cell lysate were separated on a 15% acrylamide gel. The gel was blotted to a polyvinylidene difluoride membrane as described above. The membrane was blocked as described above and incubated in a 1:200 dilution of affinity-purified Shh antiserum (see below) and then in a 1:5,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG; Jackson Immunoresearch, Inc.). Bands were visualized with the Enhanced Chemiluminescence kit (Amersham, Inc.) according to the manufacturer's instructions.

For Western blotting of mouse and chicken embryonic tissue lysates, 60 μg of each sample was separated on 15% acrylamide gels. Blotting and probing with affinity-purified Shh antiserum as well as chemiluminescence detection were carried out as described above for the COS cell material.

Immunoprecipitation. Cell lysates (*Xenopus* oocytes or COS cells) were brought to 0.5 ml with TENT (plus protease inhibitors as described above). Medium samples (OR2 or DMEM) were cleared by centrifugation at 13,000 × g for 5 min (4°C), and 10× TENT was added to a final concentration of 1× (final volume, 0.5 to 1.5 ml). The c-Myc monoclonal antibody hybridoma supernatant was added to 1/20 of the final volume. Samples were rotated for 1 h at 4°C, and then 0.1 ml of 10% (vol/vol) protein A-Sepharose CL-4B (Pharmacia, Inc.) was added. The samples were rotated for an additional 14 to 16 h. Immune complexes were washed four times with 1.0 ml of TENT. Immunoprecipitated material was eluted and denatured by boiling for 10 min in 25 μl of 1× LSB. Following centrifugation, samples were separated on 15% acrylamide gels and processed for fluorography as described previously. Samples for endo H digestion were eluted and denatured by boiling for 10 min in the provided denaturation buffer and then were digested with endo H for 1 to 2 h at 37°C. Concentrated (5×) LSB was added, and the samples were processed for electrophoresis as described above.

For immunoprecipitation with the anti-mouse Shh serum, samples (COS cell lysates and DMEM) were precleared by incubation for 1 h on ice with 3 μl of preimmune serum and then by the addition of 0.1 ml of 10% (vol/vol) protein A-Sepharose. After rotation for 1 h at 4°C, supernatants were recovered and incubated for 1 h on ice with 3 μl of depleted anti-mouse Shh serum (see below). Incubation with protein A-Sepharose, washing, elution, and electrophoresis were then performed as described above.

Immunofluorescence staining of COS cells. Twenty-four hours after transfection, the cells were transferred to eight-chamber slides (Lab-Tek, Inc.) and allowed to attach for an additional 24 h. The cells were fixed in 2% paraformaldehyde–0.1% glutaraldehyde, washed in PBS, and permeabilized in 1% Triton X-100 (28). After being washed in PBS, the cells were treated for 10 min in 1 mg of sodium borohydride per ml. The cells were incubated with the c-Myc mono-

clonal antibody hybridoma supernatant (diluted 1:10) and the affinity-purified mouse Sonic hedgehog antiserum (diluted 1:4) for 45 min, which was followed by incubation in 1:100 goat anti-mouse IgG tetramethyl rhodamine isothiocyanate (TRITC) plus 1:100 goat anti-rabbit IgG fluorescein isothiocyanate (FITC; Southern Biotechnology Associates, Inc.) for 45 min. 4′,6-Diamidino-2-phenylindole (DAPI; Sigma, Inc.) was included at 0.3 µg/ml. The slides were mounted in Slo-Fade (Molecular Probes, Inc.) and photographed on a Leitz DMR compound microscope.

Embryonic tissue dissection and lysis. Mouse forebrain, midbrain, hindbrain, lung, limb, stomach, and liver tissues from 15.5-day-postcoitum Swiss Webster embryos were dissected into cold PBS, washed several times in PBS, and then lysed by trituration and gentle sonication in LSB lacking bromophenol blue. Lysates were cleared by brief centrifugation, and protein concentrations were determined by the Bradford dye-binding assay.

To obtain chicken CNS and limb bud tissue, fertilized eggs (Spafas, Inc.) were incubated at 37°C until the embryos reached stages 20 and 25, respectively (10). By using sharp tungsten needles, dorsal and ventral pieces of the anterior CNS were obtained from the stage 15 embryos, and limb buds from the stage 25 embryos were cut into anterior and posterior halves. Tissues were lysed, and protein concentrations were determined as described above. Prior to electrophoresis of mouse and chicken proteins (see above), samples were brought to 20 µl with LSB containing bromophenol blue and boiled for 5 min.

Antibody production and purification. A PCR fragment encoding amino acids 44 to 143 of mouse Sonic hedgehog was cloned in frame into the *Eco*RI site of pGEX-2T (Pharmacia, Inc.). Transformed bacteria were induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and the fusion protein was purified on a glutathione-agarose affinity column (Pharmacia, Inc.) according to the manufacturer's instructions. Inoculation of New Zealand White rabbits, as well as test and production bleeding, was carried out at Hazleton Research Products, Inc.

To deplete the serum of antibodies against glutathione *S*-transferase (GST) and bacterial proteins, a lysate of *Escherichia coli*-transformed with pGEX-2T and induced with IPTG was coupled to Affi-Gel 10 (Bio-Rad, Inc.). The serum was incubated in batch for 2 h with the depletion matrix before centrifugation (1,000 × *g* for 5 min) and collection of the supernatant. To make an affinity matrix, purified bacterially expressed protein corresponding to the amino-terminal two-thirds of mouse Sonic hedgehog was coupled to Affi-Gel 10 (Bio-Rad, Inc.). The depleted antiserum was first adsorbed to this matrix in batch and then transferred to a column. The matrix was washed with TBST (25 mM Tris-HCl [pH 7.5], 140 mM NaCl, 5 mM KCl, 0.1% Triton X-100), and the purified antibodies were eluted with 10 bed volumes of 0.15 M glycine (pH 2.5). The solution was neutralized with 1 volume of 1 M Tris-HCl (pH 8.0) and was dialyzed against 160 volumes of PBS.

RESULTS

In vitro-translated Sonic hedgehog is proteolytically processed and glycosylated. The open reading frames of chicken and mouse *Shh* proteins encode primary translation products of 425 and 437 amino acids, respectively, with predicted molecular masses of 46.4 and 47.8 kDa (6, 34). Further examination of the protein sequences revealed a short stretch of aminoterminal residues (26 for chicken and 24 for mouse proteins) that are highly hydrophobic and are predicted to encode signal peptides. Removal of these sequences would generate proteins of 43.7 (chicken Shh) and 45.3 (mouse Shh) kDa. In addition, each protein contains a single consensus site for N-linked glycosylation (24) at residues 282 (chicken Shh) and 279 (mouse Shh). These features of the Shh proteins are summarized in Fig. 1A.

A rabbit reticulocyte lysate programmed with in vitro-translated mRNA encoding either chicken or mouse Shh synthesizes proteins with molecular masses of 46 and 47 kDa, respectively (Fig. 1B [bands labeled a]). These values are in good agreement with those predicted by examination of the amino acid sequences. To examine posttranslational modifications of Shh proteins, a preparation of canine pancreatic microsomal membranes was included in the translation reactions. This preparation allows such processes as signal peptide cleavage and core glycosylation to occur. When the Shh proteins are synthesized in the presence of these membranes, two products with apparent molecular masses of approximately 19 and 28 kDa (chicken) or 19 and 30 kDa (mouse) are seen in addition to the 46- and 47-kDa forms (Fig. 1B [bands labeled c and d]). When the material synthesized in the presence of the mem-
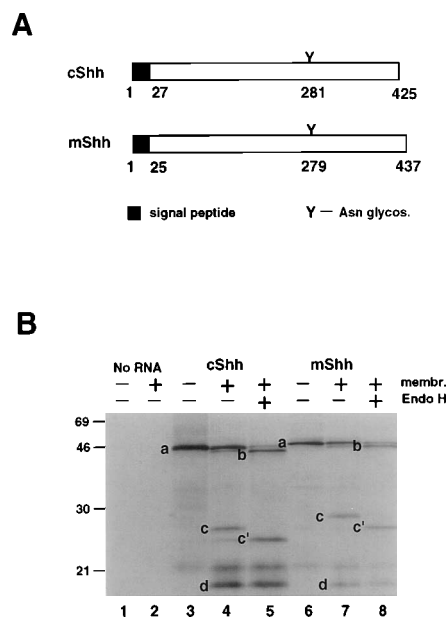

FIG. 1. (A) Schematic representations of chicken (cShh) and mouse (mShh) Shh proteins. The putative signal peptides and Asn-linked glycosylation (glycos) sites are shown. The numbers refer to amino acid positions (numbering based on the system described by Echelard et al. [6]). (B) In vitro-translated chicken and mouse Shh. Rabbit reticulocyte lysate was programmed with in vitro-transcribed chicken (lanes 3, 4, and 5) or mouse (lanes 6, 7, and 8) *Shh* RNA. Reactions were carried out in the presence (lanes 2, 4, 5, 7, and 8) or absence (lanes 1, 3, and 6) of canine pancreatic microsomal membranes (membr). Digestion with endo H was performed prior to electrophoresis (lanes 5 and 8). Control reactions without RNA are shown in lanes 1 and 2. The various Shh proteins are labeled with lowercase letters (see text for details).

branes is digested with endo H, the mobilities of the two larger proteins are increased (Fig. 1B [bands labeled b and c′]). The apparent molecular masses of the endo H-digested forms are 44 and 26 kDa for chicken Shh and 45 and 27 kDa for mouse Shh. The decrease in the molecular masses of the largest proteins synthesized in the presence of the microsomal membranes after endo H digestion (Fig. 1B [bands labeled b]) is consistent with removal of the predicted signal peptides. The mobility shift following endo H treatment indicates that N-linked glycosylation occurs and that the 26 (chicken)- and 27 (mouse)-kDa proteins contain the glycosylation sites.

The appearance of the two lower-molecular-mass bands (hereafter referred to as the processed forms) upon translation in the presence of microsomal membranes suggests that a proteolytic event in addition to signal peptide cleavage takes place. The combined molecular masses of the processed forms (19 and 26 kDa for chicken protein and 19 and 27 kDa for mouse protein) add up to approximately the predicted masses of the signal peptide-cleaved proteins (44 and 45 kDa for chicken and mouse proteins, respectively), suggesting that only a single additional cleavage occurs.

The mouse Shh protein sequence is 12 amino acid residues longer than the chicken sequence (437 versus 425 residues). Alignment of the chicken and mouse Shh protein sequences reveals that these additional amino acids are near the carboxy terminus of the protein (6). Since the larger of the processed forms differs in molecular mass by approximately 1 kDa between the two species, it appears that these peptides contain the carboxy-terminal portions of the Shh proteins. The smaller processed forms, whose molecular masses are identical, presumably consist of the amino-terminal portions.
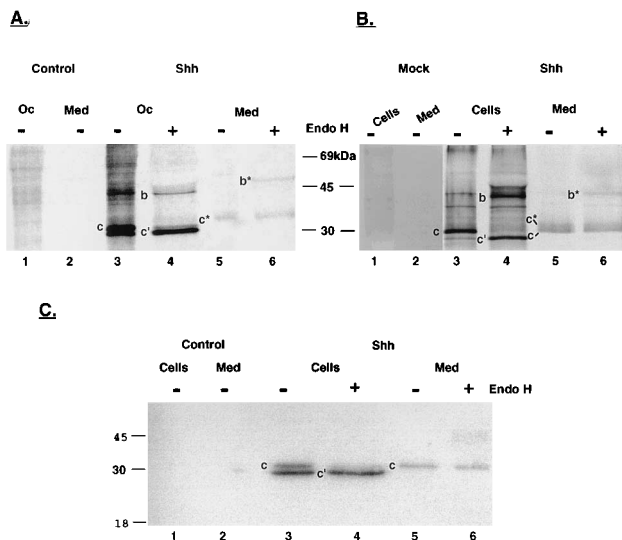
FIG. 2. (A) Expression of mouse Shh in *Xenopus* oocytes (Oc). Cell lysates (lanes 1, 3, and 4) and medium (Med) (lanes 2, 5, and 6) from ^{35}S-labeled oocytes injected with RNA encoding mouse Shh with the c-Myc epitope tag at the carboxy terminus (lanes 3 to 6) or from control oocytes (lanes 1 and 2) were analyzed by immunoprecipitation with the c-Myc monoclonal antibody. Immunoprecipitated material was digested with endo H prior to electrophoresis (lanes 4 and 6). Lanes with medium samples were exposed three times longer than lanes with oocyte samples. (B) Expression of mouse Shh in COS cells. Cell lysates (lanes 3 and 4) and medium (Med) (lanes 5 and 6) from ^{35}S-labeled COS cells transfected with a construct expressing carboxy-terminal Myc-tagged Shh were immunoprecipitated with the c-Myc antibody. Digestion with endo H was carried out prior to electrophoresis (lanes 4 and 6). Material from mock-transfected cells was also analyzed (lanes 1 and 2). Lanes with medium samples were exposed seven times longer than lanes with cell lysates. (C) Expression of mouse Shh in insect cells infected with a recombinant baculovirus. Cell lysates (lanes 1, 3, and 4) and TCA-precipitated medium (lanes 2, 5, and 6) from cultures infected with a baculovirus expressing mouse Shh with the carboxy-terminal Myc tag (lanes 3 to 6), or a control virus (lanes 1 and 2) were analyzed by Western blotting. Endo H digestion was carried out prior to electrophoresis and blotting (lanes 4 and 6).

Secretion of Shh peptides. To investigate the synthesis of Shh proteins in vivo, the mouse protein was expressed in several different eucaryotic cell types. In order to detect synthesized protein and to facilitate future purification, the carboxy terminus was engineered to contain a 25-amino-acid sequence containing a recognition site for the thrombin restriction protease, which was followed by a 10-amino-acid sequence derived from the human c-Myc protein and six consecutive histidine residues. The c-Myc sequence serves as an epitope tag allowing detection by a monoclonal antibody (9E10 [7]). The combined molecular mass of the carboxy-terminal additions is approximately 3 kDa.

X. laevis **oocytes.** Immunoprecipitation with the c-Myc antibody detects several proteins in lysates of metabolically labeled *X. laevis* oocytes injected with *Shh* mRNA. A band of approximately 47 kDa is seen, as is a doublet migrating near 30 kDa (Fig. 2A, lane 3). Treatment with endo H increases the mobility of the largest protein and resolves the doublet into a single species of approximately 30 kDa (Fig. 2A, lane 4; the bands are labeled to correspond to the in vitro-translated products [Fig. 1]). These observations parallel the behaviors seen in vitro. If allowance is made for the added mass of the carboxy-terminal additions, the largest protein would correspond to the signal peptide-cleaved form, while the doublet would represent the glycosylated and unglycosylated larger processed form. Since the epitope tag was placed at the carboxy terminus of the protein, the identity of the 30-kDa peptide as the carboxy-

terminal portion of Shh is confirmed. Failure to detect the 19-kDa species supports its identity as an amino-terminal region of the protein.

To test whether Shh is secreted by *Xenopus* oocytes, the medium in which the injected oocytes were incubated was probed by immunoprecipitation with the c-Myc antibody. A single band migrating slightly more slowly than the glycosylated larger processed form was observed (Fig. 2A, lane 5). This protein is insensitive to endo H (Fig. 2A, lane 6). This is expected, since most secreted glycoproteins lose sensitivity to endo H as they travel through the Golgi apparatus and are acted upon by a series of glycosidases (17). This enzymatic maturation of the Asn-linked carbohydrate moiety could also explain the slight decrease in mobility of the secreted larger protein versus the intracellular material. Following endo H digestion, a band with a mobility slightly lower than that of the signal peptide-cleaved protein is also seen. This suggests that some Shh protein is secreted without undergoing proteolytic processing. Failure to detect this protein in the medium without endo H digestion suggests that heterogeneity in the extent of carbohydrate modification in the Golgi apparatus prevents the material from migrating as a distict band. Resolution of this material into a single band following endo H digestion suggests that the carbohydrate structure does not mature completely in the Golgi apparatus. Structural differences between the unprocessed protein and the larger processed form could account for this observation (17).

COS cells. The behavior of mouse Shh in a mammalian cell type was investigated using transfected COS cells. Synthesis and secretion of the protein were monitored by immunoprecipitation using the c-Myc antibody. Figure 2B (lanes 3 and 4) shows that lysates of transfected COS cells contain the same Sonic hedgehog species that were detected in the injected *Xenopus* oocytes and that their behaviors following endo H digestion are also similar. Furthermore, secretion of the 30-kDa glycosylated form, as well as the characteristic insensitivity to endo H after secretion, is observed in COS cells (Fig. 2B, lanes 5 and 6). Most of the secreted protein comigrates with the intracellular, glycosylated, larger processed form; however, a small amount of protein with a slightly lower mobility is also detected in the medium. As in the *Xenopus* oocyte cultures, some Shh which has not undergone proteolytic processing is evident in the medium only after endo H digestion.

Recently, Chang et al. (5) reported similar processing of a murine Hedgehog protein, which they termed Hhg-1. Using antisera directed against amino- and carboxy-terminal regions of this protein, they detected a 19-kDa amino-terminal peptide and a 28-kDa carboxy-terminal peptide in lysates of transfected QT6 cells. However, secretion and glycosylation of the processed forms were not directly addressed in this study.

Baculovirus-infected cells. To examine the behavior of the mouse Shh protein in an invertebrate cell type and to potentially purify Shh peptides, a recombinant baculovirus was constructed which placed the *Shh* coding sequence, with the carboxy-terminal tag, under the control of the baculoviral polyhedrin gene promoter. When insect cells were infected with the recombinant baculovirus, Shh peptides could be detected in cell lysates and medium by Western blotting with the c-Myc antibody.

The Shh products detected in this system were similar to those described above. However, virtually no unprocessed protein was seen in cell lysates, nor was any detected in the medium after endo H digestion. This suggests that the proteolytic processing event occurs more efficiently in these cells than in either of the other two cell types or the in vitro translation system. A doublet corresponding to the glycosylated and un-
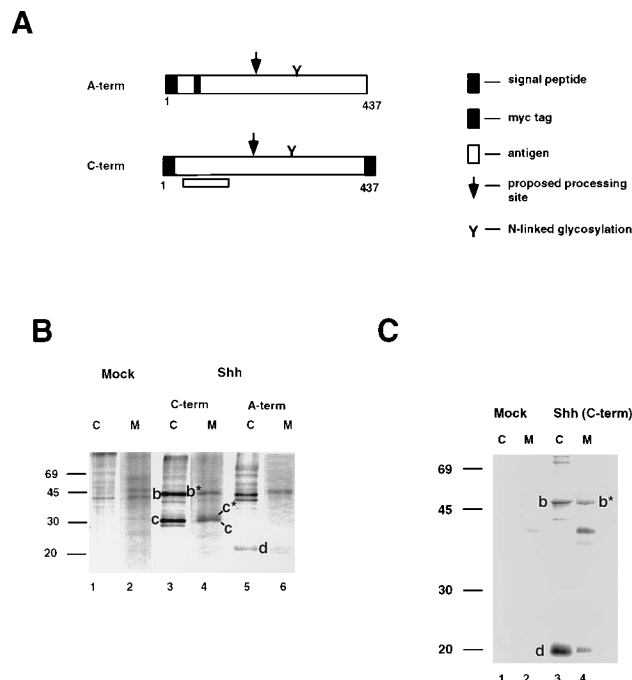
A

B

C

FIG. 3. (A) Schematic representation of Myc-tagged Shh constructs. The positions of the c-Myc epitope tags are shown, as is the predicted position of the proteolytic cleavage site. The shaded area below the carboxy-terminal tagged construct represents the region included in the GST fusion protein used to generate antisera in rabbits. A-term, amino terminal; C-term, carboxy terminal. (B) Expression of the two c-Myc-tagged Shh constructs in COS cells. COS cells were transfected with Shh expression constructs containing the c-Myc tag at the carboxy terminus (c-term [lanes 3 and 4]) or near the amino terminus (a-term [lanes 5 and 6]). ^{35}S-labeled cell lysates (C [lanes 1, 3, and 5]) and media (M [lanes 2, 4, and 6]) were analyzed by immunoprecipitation with the c-Myc antibody. Cell lysates and medium from mock-transfected cells were also analyzed (lanes 1 and 2). Lanes with medium samples were exposed five times longer than lanes with cell lysates. (C) Detection of Shh with anti-Shh serum. COS cells were transfected with the Shh construct containing the c-Myc tag at the carboxy terminus. ^{35}S-labeled cell lysates (C [lanes 1 and 3]) and media (M [lanes 2 and 4]) were analyzed by immunoprecipitation with anti-Shh serum. Mock-transfected cell lysates and medium were also analyzed (lanes 1 and 2). Lanes with medium samples were exposed eight times longer than lanes with cell lysates.

glycosylated 30-kDa forms, as well as the secreted, endo H-resistant peptide (Fig. 2C, lanes 3 to 6), is detected, as is the case with the other expression systems. However, unlike the case with the other systems, all of the secreted larger processed form appears to comigrate with the glycosylated intracellular material.

Secretion of a highly conserved amino-terminal peptide. To determine the behavior of the amino-terminal portion of the processed Sonic hedgehog protein, the c-Myc epitope tag was positioned 32 amino acids after the putative signal peptide cleavage site (Fig. 3A). When this construct was expressed in COS cells, both the full-length protein and the smaller processed form (approximately 20 kDa because of addition of the c-Myc tag) were detected by immunoprecipitation from labeled cell lysates (Fig. 3B, lane 5). However, the 20-kDa product is barely detected in the medium (Fig. 3B, lane 6). In cells transfected in parallel with the carboxy-terminal c-Myc-tagged construct, the full-length and 30-kDa products were precipitated both from cell lysates and from medium as described earlier (Fig. 3B, lanes 3 and 4). Comparison of the amounts of the amino (Fig. 3B, lane 6)- and caboxy (Fig. 3B, lane 4)-terminal peptides to the amounts of full-length (signal peptide-

cleaved) protein present in the medium suggests that the amino-terminal peptide is secreted less efficiently than the carboxy-terminal form or is less stable in the medium.

Since the amino-terminal Myc tag may affect the secretion efficiency of the smaller processed form, we examined the expression of this protein in COS cells using an antiserum directed against amino acids 44 through 143 of mouse Shh (Fig. 3A). After transfection with the carboxy-terminal Myc-tagged construct, immunoprecipitation with the anti-Shh serum detected a very low level of the 19-kDa processed form in the medium (note long exposure time) despite a strong signal in the cell lysate (Fig. 3C, lanes 3 and 4). This recapitulates the results with the Myc antibody.

To examine the subcellular localizations of Shh proteins, COS cells were transfected with the carboxy-terminal tagged Shh construct and stained with the anti-Shh serum and the Myc antibody. As shown in Fig. 4A, strong perinuclear staining characteristic of the Golgi apparatus was observed with the anti-Shh serum (Fig. 4B). The same subcellular region was also stained using the c-Myc antibody (Fig. 4C). The coincidence of staining patterns seen with the two antibody preparations is clearly shown in the double exposure (Fig. 4D). These data suggest that the low level of the 19-kDa amino-terminal peptide detected in the medium is not due to its retention in the endoplasmic reticulum, since both processed forms traffic efficiently to the Golgi apparatus.

One explanation for the failure to detect large amounts of the smaller processed form in the culture medium could be that this protein associates tightly with the cell surface or ECM. To examine this, cells were treated with the polyanionic compounds heparin and suramin. These compounds have been shown to increase the levels of some secreted proteins in culture medium, possibly by displacing them from cell surface or ECM components or by directly binding the proteins and perhaps protecting them from proteolytic degradation (3, 26, 42). As shown in Fig. 5A, the 19-kDa amino-terminal form of Shh is barely detectable in the medium of transfected COS cells (lane 3), despite its obvious presence in the cell lysate (lane 2). However, in the presence of 10 mg of heparin per ml, this peptide is readily detected in the medium (lane 5). The addition of 10 mM suramin to the medium has an even greater effect (lane 7). Since the concentrations used were those previously determined to elicit maximal responses, it is clear that suramin is more active than heparin in this assay.

The ability of heparin and suramin to increase the amount of the smaller processed form in the medium of transfected cells implies that this peptide may be tightly associated with the cell surface or ECM. As a first step toward determining which region(s) of the Shh protein may be responsible for this retention, a truncated form of mouse Shh deleted of all sequence downstream of amino acid 193 was expressed in COS cells. This protein contains all of the sequences encoded by exons one and two, as well as four amino acids derived from exon three. Since its predicted molecular mass (19.2 kDa) is very close to the observed molecular mass of the smaller processed form, the behavior of this protein would be expected to mimic that of the smaller processed form. As shown in Fig. 5A, this protein is detected at a very high level in the medium, even in the absence of heparin or suramin (compare lanes 3 and 9), and migrates at a position indistinguishable from that of the amino-terminal cleavage product generated from the full-length protein. In fact, virtually no protein is seen in the cell lysates (lanes 8, 10, and 12), suggesting nearly quantitative release of the protein into the medium. This raises the possibility that the actual amino terminally processed form may extend a short distance beyond amino acid 193 and that these
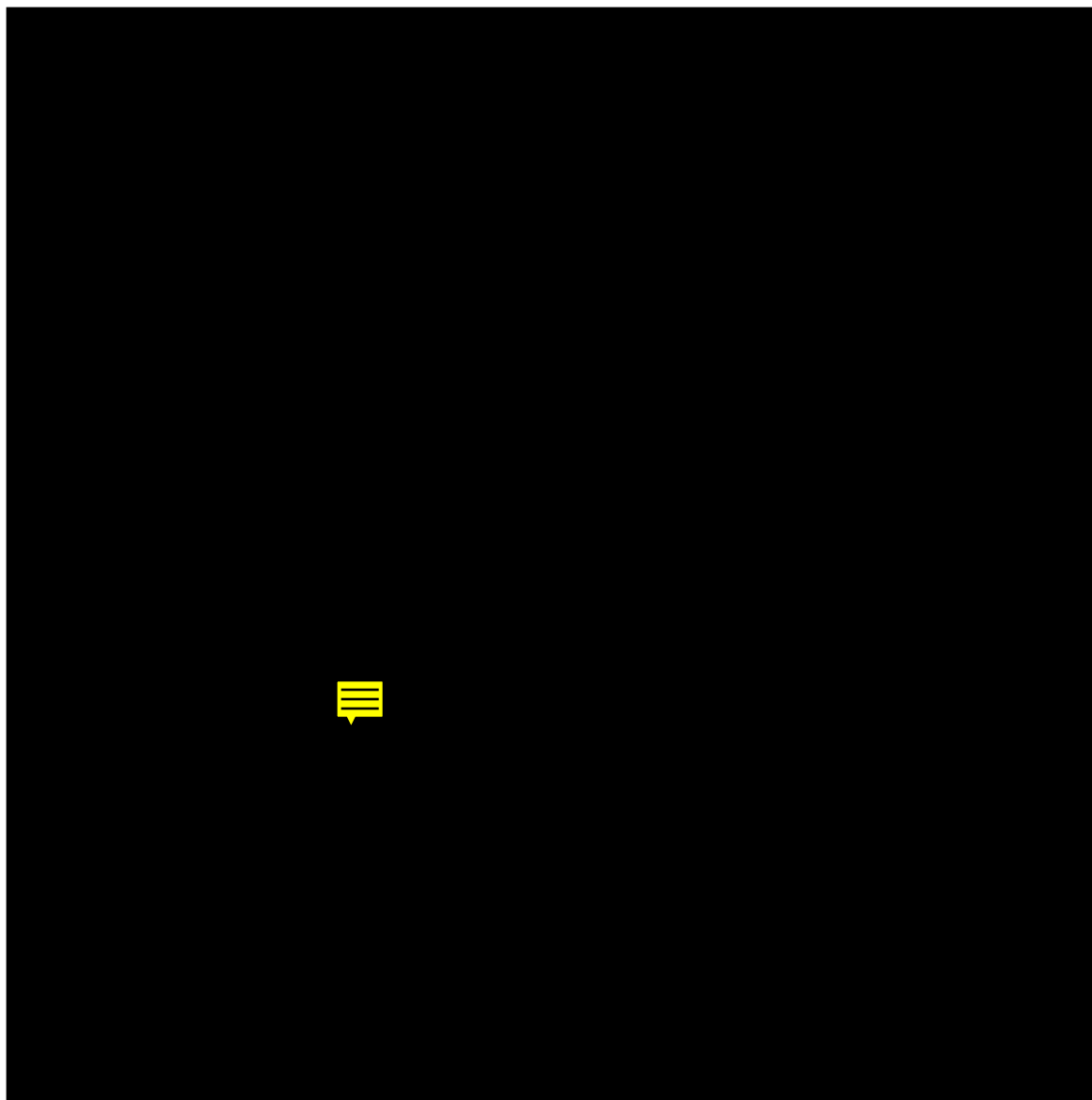
FIG. 4. (A) Schematic diagram of a carboxy-terminal Myc-tagged Shh protein. The signal peptide, c-Myc tag, glycosylation site, and putative proteolytic processing site are shown, as is the region of the protein used to raise the antiserum. Symbols are as defined in the legend to Fig. 3A. (B) Immunofluorescence detection of Shh with the Shh antiserum. COS cells transfected with the carboxy-terminal Myc-tagged Shh construct were plated on multichamber slides, fixed, and permeabilized. The cells were incubated simultaneously with Shh antiserum and the c-Myc antibody and then with FITC-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-mouse IgG. DAPI was included to stain nuclei. The same field, which is representative of all of the antibody-positive cells, is shown in panels B, C, and D. The image in this panel is a double exposure revealing DAPI and FITC staining. (C) Immunofluorescence detection of Shh with the c-Myc antibody. The image is a double exposure revealing DAPI and TRITC staining. (D) Immunoflorescence detection of Shh with the Shh serum and c-Myc antibody. The image is a triple exposure revealing DAPI, TRITC, and FITC staining.

additional amino acids contain a cell surface-ECM retention signal.

The influence of sequences located at the extreme amino and carboxy termini of mouse Shh on the behavior of the protein in transfected cells was examined using the amino terminus-specific antiserum. As shown in Fig. 5B (lane 3), expression of a mouse Shh construct lacking a signal peptide results in the accumulation of an approximately 28-kDa protein (marked by an asterisk), as well as a small amount of a protein which comigrates with the smaller processed form. This implies that correct cleavage of Shh requires targeting of the protein to the endoplasmic reticulum, since the bulk of the processed form of Shh expressed in the cytoplasm is cleaved at a new position that is approximately 9 kDa carboxy terminal to the normal cleavage site. Expression of a mouse Shh protein engineered to terminate after amino acid 428 (lacking nine carboxy-terminal amino acids [ΔCt]) results in the expected amino-terminal cleavage product; however, the efficiency of cleavage is significantly decreased compared with that seen with the wild-type protein (compare ratio of b to d in lanes 2 and 4). Therefore, sequences located at a distance from the proteolytic processing site are able to affect the efficiency of processing.

Sonic hedgehog processing in embryonic tissues. In order to
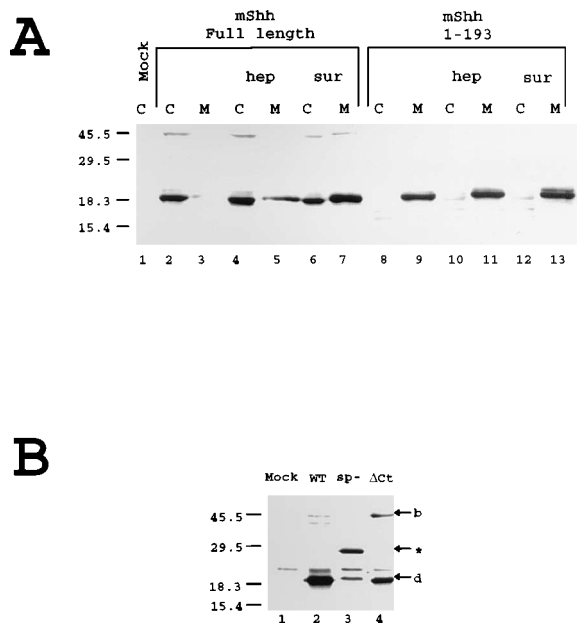
A

B

FIG. 5. (A) Effects of heparin (hep) and suramin (sur) on mouse Shh proteins. COS cells transfected with constructs expressing full-length mouse Shh (mShh [lanes 2 to 7]) or a truncated mouse Shh containing amino acids 1 through 193 (lanes 8 to 13), were incubated in 35-mm dishes with medium containing 10 mg of heparin per ml (lanes 4, 5, 10, and 11), 10 mM suramin (lanes 6, 7, 12, and 13) or no additive (lanes 2, 3, 8, and 9). Cells were lysed in LSB, and 10% of each lysate was analyzed (lanes marked C). Medium proteins were precipitated with TCA, and 50% of each sample was analyzed (lanes marked M). After electroblotting, Shh proteins were detected with the mouse Shh antiserum. A lysate of mock-transfected COS cells was included as a control (lane 1). (B) Effects of amino- and carboxy-terminal deletions on mouse Shh processing. Mock-transfected COS cells (lane 1) or COS cells transfected with constructs expressing wild-type (WT) mouse Shh (lane 2), mouse Shh lacking amino acids 1 through 20 (sp⁻ [lane 3]), or mouse Shh lacking amino acids 429 to 437 (ΔCt [lane 4]) were lysed in LSB, and 10% of each lysate (from 35-mm dishes) was processed for Western blotting. Proteins were detected with the mouse Shh antiserum. The positions of signal peptide-cleaved Shh (b), the amino-terminal form of Shh (d), and the novel form present only in cells expressing the amino terminally deleted construct (*) are indicated.
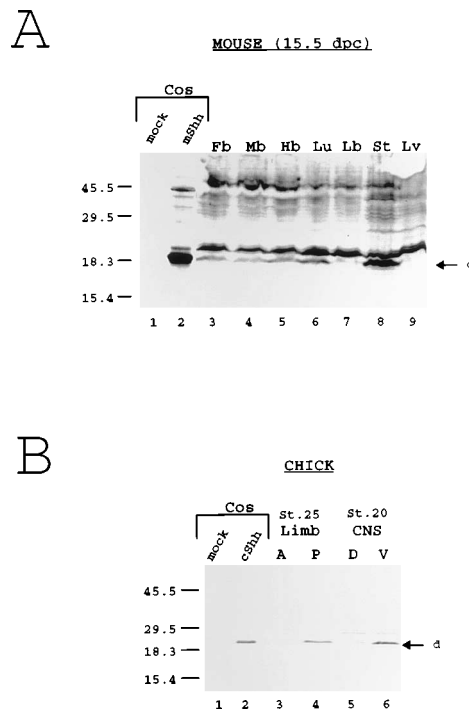
A

B

FIG. 6. (A) Shh proteins in mouse embryonic tissues. Sixty micrograms of total protein from 15.5-day-postcoitum (dpc) mouse forebrain (Fb [lane 3]), midbrain (Mb [lane 4]), hindbrain (Hb [lane 5]), lung (Lu [lane 6]), limbs (Lb [lane 7]), stomach (St [lane 8]), and liver (Lv [lane 9]) were separated by SDS-PAGE and electroblotted. Mock-transfected (lane 1) and mouse Shh (mShh)-transfected (lane 2) COS cell lysates were included for comparison. Proteins were detected with the mouse Shh antiserum. The position of the amino-terminal form of mouse Shh is indicated (d). Note that a cross-reacting band of unknown identity migrates slightly more slowly than the 19-kDa amino-terminal peptide in all tissues examined. (B) Shh proteins in chicken embryonic tissues. Sixty micrograms of total protein from anterior (A [lane 3]) and posterior (P [lane 4]) regions of limb buds from stage (st.) 25 chicken embryos and dorsal (D [lane 5]) and ventral (V [lane 6]) regions of the anterior CNS of stage 20 chicken embryos were separated by SDS-PAGE and electroblotted. Mock-transfected (lane 1) and chicken Shh (cShh)-transfected (lane 2) COS cell lysates were included for comparison. Proteins were detected with the mouse Shh antiserum. The position of the amino-terminal form of cShh is indicated (d).

determine whether the proteolytic processing of Shh observed in the different expression systems reflects the behavior of the protein in embryos, the amino terminus-specific mouse Shh antiserum was used to probe Western blots of various chicken and mouse embryonic tissues. As shown in Fig. 6A, a protein with an electrophoretic mobility identical to that of the COS cell-synthesized amino-terminal form is detected at a substantial level in the stomach and lung tissue and at a markedly lower level in the forebrain, midbrain, and hindbrain tissues of 15.5-day-postcoitum mouse embryos. These tissues have all been shown to express *Shh* RNA (2). The 19-kDa peptide is not detected in liver or late limb tissues, which do not express *Shh* RNA (2). Thus, the proteolytic processing of Shh observed in cell culture also occurs in embryonic mouse tissue.

We were able to take advantage of the cross-reactivity of the amino terminus-specific mouse Shh antiserum with chicken Shh protein to examine expression of Shh in chicken embryonic tissue. As shown in Fig. 6B, the antiserum detects the 19-kDa amino-terminal form of chicken Shh in transfected COS cells, as well as in two tissues which have been shown by whole-mount in situ hybridization and antiserum staining to express high levels of *Shh* RNA and protein, i.e., the posterior region of the limb bud and the ventral region of the anterior CNS (9, 25, 34). Therefore, the expected proteolytic processing

of Shh occurs in chicken embryonic tissues, and diffusion of the 19-kDa protein does not extend into the anterior limb buds and dorsal CNS.

DISCUSSION

Hedgehog processing. In this report, we have demonstrated that the mouse and chicken *Shh* genes encode secreted glycoproteins which undergo additional proteolytic processing. We have shown that this processing occurs in an apparently similar fashion in a variety of cell types, suggesting that it is a general feature of the Shh protein and not unique to any particular expression system and, moreover, that it is a normal aspect of mouse and chicken development. For mouse Shh, we have demonstrated that both products of this proteolytic processing are secreted. These observations are summarized in Fig. 7. The 19-kDa amino-terminal form accumulates to a lower level in the medium than the 27-kDa carboxy-terminal form. This could be due to inefficient secretion or rapid turnover of this species once it is secreted. Alternatively, the 19-kDa amino-terminal peptide may associate with the cell surface or extracellular matrix components, making it difficult to detect in the medium. The latter explanation is favored, since heparin and,
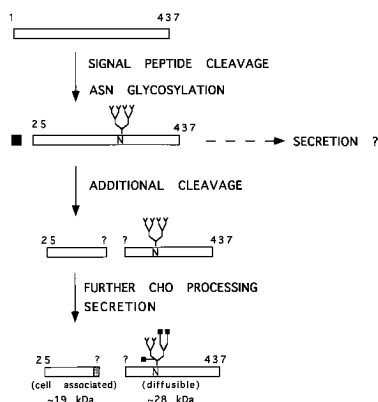
FIG. 7. Schematic diagram of mouse Shh processing. Illustrated are cleavage of the signal peptide (filled box), glycosylation at the predicted Asn residue (N), and the secondary proteolytic cleavage. The question marks indicate that the precise site of proteolytic cleavage has not been determined. The shaded box at the carboxy terminus of the 19-kDa peptide represents the putative cell-ECM retention signal. The different symbols representing the carbohydrate moiety represent maturation of this structure in the Golgi apparatus. The broken arrow leading from the signal peptide-cleaved protein indicates that secretion of this species may be an artifact of the incomplete proteolytic processing of Shh seen in *Xenopus* oocytes and COS cells. The numbers refer to amino acid positions according to Echelard et al. (6).

to a greater extent, suramin significantly increase the amount of the 19-kDa form in the medium of transfected cells. Moreover, a truncated mouse Shh protein, which is likely to correspond very closely to the amino-terminal form, accumulates to a high level in tissue culture medium even in the absence of heparin or suramin, suggesting that the retention of the 19-kDa peptide is due to sequences at its extreme carboxyl terminus which are missing in the truncated form.

The insensitivity of the secreted, larger form to endo H is a common feature of secreted glycoproteins. During transit through the Golgi apparatus, the Asn-linked carbohydrate moiety is modified by a series of specific glycosidases (for reviews, see references 17 and 46). These modifications convert the structure from the immature high-mannose to the mature complex type. At one step in this process, a Golgi enzyme, α-mannosidase II, removes two mannose residues from the complex, rendering it insensitive to endo H (17).

The biochemical behavior of mouse Shh appears to be quite similar to that described for the *Drosophila* Hedgehog (HH) protein (21, 22, 44). In vitro translation of *Drosophila hh* mRNA, in the presence of microsomes, revealed products with molecular masses corresponding to that of full-length protein, as well as to the product expected after cleavage of the predicted internal (type II) signal peptide (22). Interestingly, no additional processed forms were observed. However, such forms could have been obscured by breakdown products migrating between 20 and 30 kDa. When an RNA encoding a form of the protein lacking the carboxy-terminal 61 amino acids was translated, no breakdown products were seen; however, there is still no evidence of the proteolytic processing observed with mouse Shh. We observed a similar phenomenon. A reduction in the extent of proteolytic processing is seen when a mouse Shh protein lacking 9 carboxy-terminal amino acids is expressed in COS cells. This suggests that sequences at the carboxy termini of Hedgehog proteins act at a distance to influence the efficiency of processing.

Recently, Lee et al. (21) described the biochemical behavior of the *Drosophila* HH protein. Using region-specific antisera, they detect similar processed forms of HH in embryonic tis-

sues, thus confirming studies in which processing of HH was observed in embryos forced to express high levels of HH from a heat shock promoter (44). Thus, *Drosophila* HH is processed to yield a 19-kDa amino-terminal peptide and a 25-kDa carboxy-terminal peptide. Furthermore, Lee et al. concluded that the production of the processed forms occurs via an autocatalytic mechanism and identified a conserved histidine residue (at position 329, according to Lee et al. [21]) which is required for self-cleavage of HH protein in vitro and in vivo. The significance of the proteolytic processing is demonstrated by the inability of HH proteins incapable of self-processing—either because of mutation of this histidine residue or because of truncation of sequences at the extreme carboxy terminus—to carry out HH functions in *Drosophila* embryos.

Our studies of the biochemical behavior of mouse and chicken Shh proteins correlate well with the *Drosophila* studies of Lee et al. (21) in that we demonstrate similar proteolytic processing of endogenous vertebrate proteins in embryonic tissues. Furthermore, we demonstrate that the efficiency of processing depends on sequences located at the extreme carboxy terminus of mouse Shh. Interestingly, we have also shown that the specificity of mouse Shh cleavage may depend on targeting of the protein to the secretory pathway, since a form lacking a signal peptide is processed into an approximately 28-kDa amino-terminal form. A similar protein is observed as the predominant species when we attempt to express full-length mouse Shh in bacteria (data not shown). Lee et al. (21) have demonstrated that two zebra fish Hedgehog proteins undergo proteolytic processing when translated in vitro, even in the absence of microsomal membranes. The electrophoretic mobilities of the processed peptides are consistent with cleavage occurring at a position similar to that of the *Drosophila* HH cleavage site. Furthermore, they showed that the cleavage fails to occur if the conserved histidine residue is mutated, arguing for an autoproteolytic mechanism similar to that of the *Drosophila* protein. However, we do not detect processing of mouse or chicken Shh protein translated in vitro unless microsomal membranes are included. Therefore, it is possible that correct proteolytic processing of vertebrate Hedgehog proteins is dependent on specific incubation conditions or may require cellular factors in addition to Shh itself.

An additional correlation between the work presented here and that of Lee et al. (21) concerns the different behaviors of the amino (smaller)- and carboxy (larger)-terminal forms of the Hedgehog proteins. We have presented evidence that the 27-kDa carboxy-terminal form diffuses more readily from expressing cells than the 19-kDa amino-terminal form, which seems to be retained near the cell surface. The polyanions heparin and suramin appear capable of releasing the amino peptide into the medium. Similarly, the amino-terminal form of *Drosophila* HH is more closely associated with the RNA expression domain in embryonic segments than is the carboxy-terminal form, and the amino-terminal form binds to heparin agarose beads. Therefore, the distinct behaviors of the different Hedgehog peptides have been conserved across phyla.

The observed molecular masses of the amino-terminal forms of mouse and chicken Shh proteins and *Drosophila* HH are between 19 and 20 kDa. Therefore, the predicted secondary proteolytic cleavage site would be located near the border of the sequences encoded by the second and third exons. Interestingly, this region marks the end of the most highly related part of the Hedgehog proteins. The amino-terminal form would contain the most highly conserved portion of the protein. In fact, the amino acids encoded by exons one and two (exclusive of sequences upstream of the putative signal peptide cleavage sites) share 69% identity between *Drosophila* HH and
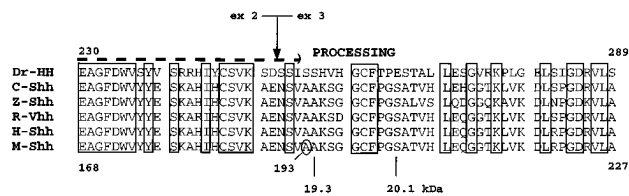
FIG. 8. Alignment of *Drosophila* HH and vertebrate Shh proteins in the vicinity of the exon 2-exon 3 border. The numbers above the aligned sequences refer to *Drosophila* HH positions (according to Lee et al. [22]). The numbers beneath the aligned sequences refer to mouse Shh positions (according to Echelard et al. [6]). The arrow indicates the exon 2-exon 3 border. The dashed line ending in a parenthesis indicates the extent of the *Drosophila* HH deletion (to I^{254}), which is still processed normally (21). The circled alanine residue (position 193) is the carboxy-terminal amino acid of the truncated mouse Shh protein which is likely to contain a short carboxyl truncation preventing cell or ECM retention of this peptide (Fig. 5). Amino acids shared by all sequences are boxed. The shaded region labeled processing is likely to contain the proteolytic processing site (see Discussion). Cleavage within this area would yield a mouse Shh amino-terminal product with a molecular mass of between 19.2 and 20.1 kDa, as indicated beneath the shaded region. Dr-HH, *Drosophila* HH (22); C-Shh, chicken Shh (34); Z-Shh, zebra fish Shh (18); R-Vhh, rat Vhh (35); H-Shh, human Shh (23); M-Shh, mouse Shh (6); ex, exon.

mouse Shh and 99% identity between chicken Shh and mouse Shh. Amino acid identity in the region encoded by the third exon is much lower, i.e., 30% identity between mouse and *Drosophila* proteins and 71% identity between mouse and chicken proteins (6).

However, the boundary between sequences encoded by exons 2 and 3 is unlikely to be the actual proteolytic processing site, since a *Drosophila* HH protein containing a large deletion which extends three amino acids beyond this boundary is still cleaved at the expected position in vitro (21). Moreover, our analysis of an amino-terminal mouse Shh peptide truncated at amino acid 193 (the fourth amino acid encoded by exon 3) suggests that normal cleavage must occur downstream of this position. Close examination of Hedgehog protein sequences reveals that strong sequence conservation between the *Drosophila* and vertebrate proteins continues for only a short distance into the third exon. If it is assumed that cleavage will generate an amino-terminal product with a molecular mass of no greater than 20 kDa, given the resolution of our analysis, all of the data would indicate that cleavage occurs at 1 of the 10 amino acids within the shaded region shown in Fig. 8 (mouse Shh positions 194 to 203, according to Echelard et al. [6]).

Hedgehog signalling. In order to satisfy the criteria for intercellular signalling, Hedgehog proteins must be detected outside their domains of expression. This has been clearly demonstrated for *Drosophila* HH. Using region-specific antisera, Lee et al. (21) showed that both the amino and carboxy-terminal forms of HH are present outside the domain of RNA expression. Using an antiserum raised against nearly full-length HH protein, Tabata and Kornberg (43) also detected the protein in stripes that are slightly wider than the RNA expression domain, and they detected HH protein in a stripe of cells just anterior to the border of the RNA expression domain in wing imaginal discs. Similarly, Taylor et al. (48) detected HH protein in discrete patches within cells adjacent to those expressing the *hh* RNA in embryonic segments by using an antiserum directed against an amino-terminal portion of HH which, based on the proteolytic processing data (21, 43), is not likely to recognize the larger of the two processed forms.

The detection of HH beyond cells expressing the *hh* gene is consistent with the phenotype of *hh* mutant flies. In these animals, cellular patterning in each embryonic parasegment is disrupted, resulting in an abnormal cuticular pattern reminis-

cent of that seen in *wg* mutants. Further analysis has revealed that the loss of *hh* gene function leads to loss of *wg* expression in a thin stripe of cells just anterior to the *hh* expression domain (14). This suggests that HH acts to maintain *wg* expression in neighboring cells. The observation that ubiquitously expressed HH leads to ectopic activation of *wg* supports this model (44). In addition to these genetic studies, there is also indirect evidence that HH acts at a distance from its site of expression to influence patterning of the epidermis (12).

The apparent effect of *Drosophila* HH on neighboring cells, as well as on those located at a distance from the site of *hh* expression, is reminiscent of the influence of the notochord and floor plate on the developing vertebrate CNS and somite and of the ZPA in the limb. The notochord (a site of high-level *Shh* expression) induces the formation of the floor plate in a contact-dependent manner, while the notochord and floor plate (another area of strong *Shh* expression) are both capable of inducing motor neurons at a distance (31, 51), and both produce a diffusible signal which ventralizes the somite (8). Moreover, ZPA activity is required not only for patterning cells in the extreme posterior of the limb bud where *Shh* is transcribed, but also a few hundred micrometers anterior of this zone. Since we have shown that there are two forms of Shh, it is tempting to speculate that the smaller, amino-terminal form, which appears to be more poorly secreted, to be less stable, or to be retained at the cell surface or in the ECM, may act locally. In contrast, the larger, carboxy-terminal peptide could function at a distance. In this way, Shh peptides may mediate distinct signalling functions in both the CNS and limbs. Alternatively, the carboxy-terminal peptide may be necessary only for proteolytic processing, with all signalling activity residing in the amino-terminal peptide. Clearly, further work must address the activities encoded by the appropriate amino- and carboxy-terminal peptides, as well as their in vivo distributions, to allow a more complete picture of Shh function to emerge.
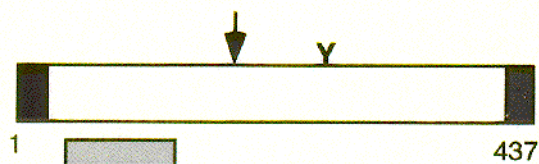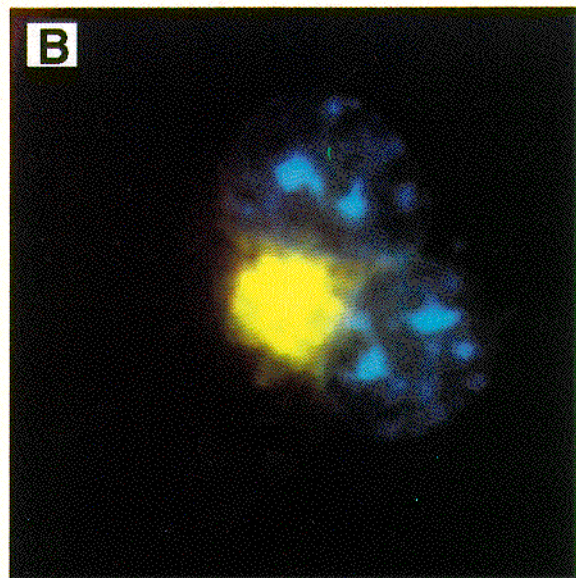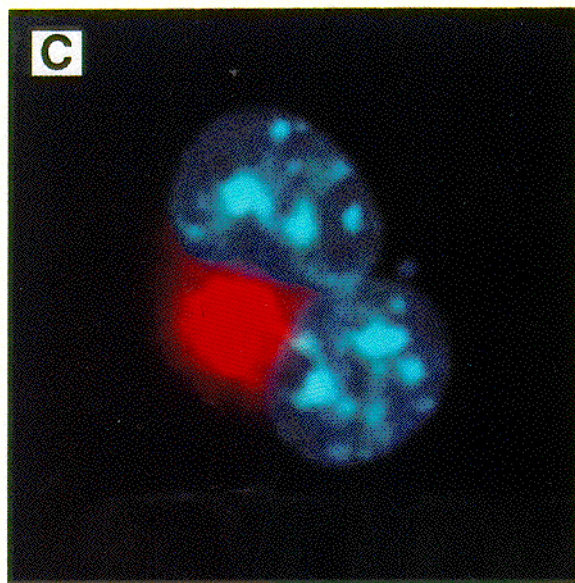
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